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Loss of Smad4 in Colorectal Cancer Cells Promotes CCL15 Expression to Recruit CCR1+ Myeloid Cells and Facilitate Liver Metastasis

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Short title: Loss of Smad4 promotes CCL15 to recruit CCR1+ cells

Abbreviations:
BMDC, bone marrow-derived cell; BMP, bone morphogenetic proteins; COPD, chronic obstructive pulmonary disease; CRC, colorectal cancer; DFS, disease-free survival; FAP, familial adenomatous polyposis; HDAC, histone deacetylase; IHC, immunohistochemistry; LOH, loss of heterozygosity; MDSC, myeloid-derived suppressor cells; MMP, matrix metalloproteinase; MPO, myeloperoxidase; RACE, rapid amplification of cDNA ends; SBE, Smad-binding element; TIE, TGF-β-inhibitory element; TGF-β, transforming growth factor-β; VEGFR1, vascular endothelial growth factor receptor 1

A list of how each author was involved with the manuscript

Yoshiro Itatani; acquisition of data, analysis and interpretation of data.
Kenji Kawada; study concept and design, analysis and interpretation of data, drafting of the manuscript, obtained funding.
Teruaki Fujishita; technical, or material support.
Fumihiko Kakizaki; technical, or material support.
Hideyo Hirai; critical revision of the manuscript for important intellectual content.
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Masayoshi Iwamoto; technical, or material support.
Susumu Inamoto; technical, or material support.
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Yoshiharu Sakai; study concept and design, technical, or material support.
Makoto Mark Taketo; study concept and design, study supervision, drafting of manuscript, obtained funding.

Conflict of interest

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Abstract

BACKGROUND & AIMS: Loss of the tumor suppressor Smad4 is correlated with progression of colorectal cancer (CRC). In mice, colon tumors expressed CCL9 and recruit CCR1+ myeloid cells, which facilitate tumor invasion and metastasis by secreting matrix metalloproteinase (MMP) 9.

METHODS: We used human CRC cell lines to investigate the ability of Smad4 to regulate expression of CCL15, a human ortholog of mouse CCL9. We employed immunohistochemistry to compare levels of CCL15 and other proteins in 141 samples of human liver metastases.

RESULTS: In human CRC cell lines, knockdown of *SMAD4* increased CCL15 expression, whereas overexpression of Smad4 decreased it. Smad4 bound directly to the promoter region of *CCL15* gene to negatively regulate its expression; transforming growth factor-β (TGF-β) enhanced binding of Smad4 to the *CCL15* promoter and transcriptional repression. In livers of nude mice, Smad4-deficinet human CRC cells upregulated CCL15 to recruit CCR1+ cells and promote the metastatic colonization. Analysis of clinical specimens showed a strong inverse correlation between levels of CCL15 and Smad4; metastases that expressed CCL15 contained 3-fold more CCR1+ cells than those without CCL15. Patients with CCL15-expressing metastases showed significantly shorter times of disease-free survival (DFS) than those with CCL15-negative metastases. CCR1+ cells in the metastases expressed the myeloid cell markers CD11b and myeloperoxidase, and also MMP9.

CONCLUSIONS: In human CRC cells, loss of Smad4 leads to upregulation of CCL15 expression. Human liver metastases with CCL15 expression contain higher numbers CCR1+ cells and these patients are associated with shorter DFS. Therapeutics that block CCL15 recruitment of CCR1+ cells may prevent metastasis of CRC to liver.

Keywords: colon cancer; carcinoma; signal transduction; chemokine

Introduction

Colorectal cancer (CRC) is one of the leading causes of cancer-related deaths worldwide. Although most CRC patients without metastases can be cured by surgical resection of the primary tumors, metastases to the liver are found in ~30% of CRC cases within 5 years.[1] When feasible, complete surgical resection is performed for the liver metastases, which leads to a 5-year survival rate ranging 30–37%.[2,3] Recurrence after resection of liver metastasis occurs in 60% of patients, within two years in most cases.[4,5] Therefore, it is imperative that we develop adjuvant drugs to prevent postoperative metastasis.

SMAD4 encodes a key molecule that mediates transforming growth factor-β (TGF-β) superfamily signaling, activated by TGF-β, activins, and bone morphogenetic proteins (BMPs). The *SMAD4* gene is located in human chromosome 18q21, a region with frequent genetic loss of heterozygosity (LOH) in CRC.[6-8] Moreover, loss of Smad4 protein is strongly correlated with progression in the primary and metastatic CRC.[9,10] The expression level of Smad4 is a prognostic marker in CRC. Namely, the patients whose CRCs express high Smad4 levels show significantly better survival than those with low Smad4 levels.[11]

We previously showed that in *cis-Apc*$^{+/\Delta 716}$ *Smad4*$^{+/-}$ (*Apc/Smad4*) mice that develop invasive intestinal adenocarcinomas, a C-C chemokine CCL9 is secreted from the CRC epithelium, which recruits myeloid cells expressing its receptor CCR1.[7,15] Recent studies in some mouse models have also revealed that bone marrow-derived cells (BMDCs) play critical roles in cancer invasion and metastasis.[12-14] These BMDCs expressing markers of myeloid cell (CD11b) and granulocyte (Gr-1) create microenvironment suitable for cancer cell survival, invasion and metastasis through production of matrix metalloproteinases (MMPs), formation of the premetastatic niche and/or suppression of immune reactions. In *cis-Apc/Smad4* mice, CCR1+ myeloid cells produce MMP9, allowing cancer cells to invade surrounding stromal tissues at the invasion front. In addition, using a mouse model of CRC liver metastasis, we have also demonstrated that

mouse and human CRC cell lines that secrete mouse CCL9 (mCCL9) and human CCL15 (hCCL15; a human ortholog of mCCL9), respectively, can recruit CCR1+ myeloid cells around metastatic foci of the liver.[16] The accumulation of CCR1+ cells is prevented by inhibition of the mCCL9/hCCL15–CCR1 signaling with CCR1 antagonist, knocking-down of *mCCL9/hCCL15* gene, or introducing the *CCR1* knockout mutation.[15,16] Importantly, invasion and metastasis of the cancer cells is suppressed by preventing CCR1+ cell accumulation.[17] Therefore, targeting the recruitment of CCR1+ myeloid cells can be an effective prevention strategy against CRC metastasis in the liver. However, it has not been investigated rigorously whether CCR1+ myeloid cells are accumulated also in human CRC specimens of liver metastasis. It is yet to be established whether expression of CCL15 has a correlation with progression of CRC. Likewise, it remains to be elucidated how human CRC cells express CCL15 at the molecular level.

Here we report that, in human clinical specimens, CCR1+ myeloid cells often accumulate around liver metastases of CRC that express CCL15. Importantly, patients with CCL15-expressing liver metastases showed significantly shorter disease-free survival (DFS) after curative liver resection than those with CCL15-negative metastases. We also show that Smad4 binds directly to the promoter region of *CCL15* gene and negatively regulates its expression through TGF-β family signaling. In a mouse model of CRC liver metastasis, we demonstrate that loss of Smad4 in human CRC promotes expression of CCL15 to recruit CCR1+ cells and facilitates liver metastasis. Taken together, these results suggest that blocking the CCL15–CCR1 axis can be an efficacious therapeutic strategy to prevent metastatic CRC in the liver.

Materials and Methods

Patients population

For the analysis of CRC liver metastases, a total of 141 liver metastases were obtained from 67 operations of 55 patients undergoing partial liver resection at Kyoto University Hospital between January 2006 and December 2010. Liver tumors were confirmed to be CRC liver metastases by pathological examinations. For the analysis of primary CRC, a total of 49 primary CRC were obtained; 27 from the patients with liver metastasis and 22 from stage I CRC patients without metastasis. This study protocol was approved by the institutional review board of Kyoto University, Kyoto, Japan, and patients provided their consents for the sample use and data analysis.

Cell lines and reagents

AA/C1 cells were kindly provided from Dr. C. Paraskeva (University of Bristol).[18] HT29, Colo205, LoVo, DLD-1 and HCT116 cells were supplied from American Type Culture Collection. A detailed list of the antibodies used can be found in Supplementary Table S1. TGF-β1, BMP4 and activin-A were purchased from Peprotech, and LY2157299 and LDN193189 were from Wako. Trichostatin A was from SIGMA.

Immunohistochemistry (IHC) analysis

Formalin-fixed, paraffin-embedded sections were stained with respective antibodies (Supplementary Table S1) by the avidin-biotin immunoperoxidase method. To quantify the densities of CCR1+ cells, sections were analyzed using ImageJ software. Measurements were recorded as the number of CCR1+ cells at the margin of liver metastases [5 to 8 fields (0.1mm^2) analyzed per liver metastasis]. For the staining of Smad4 and CCL15, we interpreted a sample as positive when > 10% of the tumor cells were stained with the respective antibodies.

In vivo experimental metastasis studies

We injected 3.0 x 10^6 of cancer cells into the spleen of eight-week old female KSN nude mice under anesthesia. The spleen was resected 1 min after tumor injection to prevent splenic tumor formation that often interfered bioluminescence imaging of liver metastatic tumors. For *in vivo* bioluminescence imaging, we injected 2 mg of D-luciferin (VivoGlo luciferin, Promega) intraperitoneally into anesthetized tumor-bearing mice 15 min before imaging. Bioluminescence from the luciferase-expressing tumor cells was scored at days 7, 14, 21 and 28 post-injection, using a Xenogen IVIS system (Xenogen Corporation) as previously described.[16] All animal experiments were approved by the Animal Care and Use Committee of Kyoto University.

Statistical analysis

All values are expressed as means ± SD. The statistical significance of differences was determined by Mann-Whitney U test, chi-square test or Student's *t*-test. All analyses were two-sided, and a *P* value of $< .05$ was considered statistically significant. Relationships between variables were determined by Pearson's correlation coefficients. Statistical analyses were performed using the SPSS software, version11.50 (SPSS Inc).

Results

Expression level of CCL15 is inversely correlated with that of Smad4 in human CRC cell lines.

Loss of Smad4 expression is associated with advanced stages of CRC and frequently observed in metastatic lesions.[9,10] We previously found that colon tumors of *Apc/Smad4*-deficient mice expressed CCL9 to promote tumor invasion and metastasis.[15] Therefore, we hypothesized that expression of its human ortholog CCL15 is also regulated by the TGF-β family signaling through Smad4 in CRC. We first determined the expression levels of both Smad4 and CCL15 in human CRC cell lines. LoVo, DLD-1 and HCT116 have wild-type Smad4 with mutant TGF-β type II receptor (TGF-βRII),[19,20] while HT29 and Colo205 lack Smad4 with wild-type TGF-βRII (Supplementary Table S4).[21,22] AA/C1 is a premalignant adenomatous cell line isolated from a patient with familial adenomatous polyposis (FAP).[18] A western blot analysis showed that Smad4-deficient cell lines HT29 and Colo205 constitutively expressed high levels of CCL15. Among the Smad4-expressing cell lines, on the other hand, LoVo expressed a low level of CCL15, whereas DLD-1, HCT116 and adenoma AA/C1 hardly expressed it (Fig. 1A). We also determined the level of mRNA for *CCL15* by quantitative RT-PCR, and found a high level in HT29, and moderate levels in Colo205 and LoVo, while very little in DLD-1 and HCT116, as well as in AA/C1 (Fig. 1B). We detected expression of other Smad family members, but their levels did not affect that of CCL15 (Fig. 1A).

Smad4 negatively regulates expression of CCL15

To further investigate the relationship between Smad4 and CCL15 expression, we performed RNA interference experiments. We introduced two siRNA constructs targeting *SMAD4* (referred as si*SMAD4*#1 and si*SMAD4*#2) into Smad4-expressing cell lines, and confirmed that both si*SMAD4* RNA species significantly decreased expression of Smad4 protein in these cell lines (Fig. 1C). We also confirmed that these siRNAs did not affect expression of other Smad members (Fig. 1C). In these Smad4-expressing cell lines,

Smad4 can induce luciferase from the SBE4-Luc reporter construct that contains four Smad-binding elements (SBEs) (Supplementary Fig. S2A). Importantly, the mRNA level of *CCL15* was significantly increased in these cell lines when *SMAD4* was knocked-down by si*SMAD4* as determined by quantitative RT-PCR (Fig. 1D). The expression level of CCL15 protein was also increased after treatment with si*SMAD4* (Supplementary Fig. S1A).

We next investigated whether exogenous expression of Smad4 reduced the CCL15 level in Smad4-deficient cell lines. When cMyc-tagged Smad4 was stably overexpressed in HT29 and Colo205 by lentiviral transduction, expression of CCL15 was significantly decreased in both protein and mRNA levels compared with that in the control cells (Fig. 1E and 1F). In TGF-β signaling, ligand binding activates TGF-β receptors and phosphorylates Smad2/3 to form heteromeric complex with Smad4 which translocates into the nucleus and regulates target gene transcription.[23,24] In our experiment using Smad4-expressing Colo205 cells, addition of TGF-β1 caused phosphorylation of Smad2 (Supplementary Fig. S1B) and formation of the Smad2/3/4 complex (Supplementary Fig. S1C). Furthermore, we confirmed that the TGF-β signaling was recovered by transduction of Smad4 in HT29 and Colo205 cells as assessed by the SBE4-Luc luciferase reporter assay (Supplementary Fig. S2B). In the Smad4-expressing HT29 and Colo205 cells, addition of TGF-β1 further reduced CCL15 mRNA and protein levels compared with those in the non-treatment control (Fig. 1E and 1F). These results indicate that Smad4 negatively regulates CCL15 expression, whereas loss of Smad4 promotes CCL15 expression in CRC cell lines.

Smad4 is one of the key transcription factors of the TGF-β superfamily signaling. Not only TGF-β, but also BMP and activin signaling appears to be involved in the progression of CRC.[25] Thus, we investigated which particular signaling played major roles in *CCL15* regulation. First, we determined the endogenous expression levels of TGF-β superfamily ligands in CRC cell lines, and found TGF-β1 and BMP4 at significant levels (Supplementary Fig. S1D). We then examined Smad4-expressing HT29 and Colo205

cells for signal responses with TGF-β1, BMP4 and activin-A. Stimulation with 10 ng/ml of TGF-β1 or BMP4 suppressed *CCL15* expression, while activin-A did not (Supplementary Fig. S1E). In LoVo cells that express Smad4 endogenously, stimulation with BMP4 inhibited expression of *CCL15*, whereas TGF-β1 and activin-A had no effect (Supplementary Fig. S1F). As anticipated, TGF-β1 and TGF-β receptor inhibitor (LY2157299) phosphorylated and de-phosphorylated Smad2, respectively, in Smad4-expressing HT29 cells. Likewise, BMP4 and BMP receptor inhibitor (LDN193189) affected phosphorylation of Smad1/5/8 (Supplementary Fig. S1G). Furthermore, treatment with an inhibitor against either receptor increased *CCL15* expression (Supplementary Fig. S1H), indicating that both TGF-β signaling and BMP signaling down-regulate *CCL15* expression through Smad4.

Smad4 binds directly to the human *CCL15* promoter and inhibits transcription

As shown in Fig. 1, knockdown of *SMAD4* increased the expression level of *CCL15*, whereas its overexpression decreased it. Moreover, TGF-β1 and BMP4 binding to their cognate receptors strengthened the inhibition. Therefore, we hypothesized that the canonical TGF-β signaling directly inhibited transcription of *CCL15* gene through Smad4. Sequence analysis of the promoter/enhancer region of human *CCL15* gene revealed four SBEs (CAGAC) at positions starting –469, –424, –114 and +94 counted from the transcription start site (Fig. 2A, top). In addition, we found a stretch of “GCTTGGC” at the position starting –31, which is similar to the TGF-β–inhibitory element (TIE) reported in the *c-Myc* and *Stromelysin-1* promoters (Fig. 2A, top).[26–28] To test whether binding of Smad4 suppressed transcription from the *CCL15* promoter, we performed luciferase reporter assays. We constructed a luciferase reporter plasmid using the –500 to +100 region that included four SBEs and one TIE-like element (Fig. 2A, bottom). With this reporter, *SMAD4* knockdown in Smad4-expressing cell lines caused up-regulation of the *CCL15* promoter activity (Fig. 2B). On the other hand, Smad4 transduction into Smad4-deficient cell lines down-regulated the promoter activity (Fig. 2C). To these cells,

addition of TGF-β1 or BMP4 further reduced the *CCL15* promoter activity compared with that in the non-treatment control, while both inhibitors up-regulated it (Fig. 2C; Supplementary Fig. S2C and S2D).

To determine which element of four SBEs or TIE is critical in *CCL15* regulation, we used a series of deletion constructs of the *CCL15* promoter that lacked the respective SBE or TIE, followed by the luciferase gene (Fig 2D left). When transfected with the constructs that carried deletion of the 3rd SBE (Δ3rd SBE) and TIE (ΔTIE), luciferase activities were significantly up-regulated compared with that of wild-type *CCL15*-Luc reporter transfection, indicating that the 3rd SBE and TIE are critical for the negative regulation of *CCL15* expression (Fig 2D middle and right).

To test whether Smad4 binds directly to SBEs and/or TIE in the *CCL15* promoter, we performed ChIP-PCR assays using an anti-Smad4 antibody. A quantitative PCR analysis of the Smad4-immunoprecipitates showed significant enrichment of the 3rd SBE and TIE region compared with that in the control (Fig. 2E and Supplementary Fig. S2E). We also evaluated the effects of TGF-β1 stimulation on the Smad4-binding to *CCL15* promoter using Smad4-expressing HT29 cells. Addition of TGF-β1 significantly increased the recruitment of Smad4 to the 2nd and 3rd SBEs and TIE region of the *CCL15* promoter (Fig. 2F). Collectively, these results demonstrate that Smad4 binds directly to the *CCL15* promoter and inhibits its transcription in CRC cell lines.

Smad4-deficient CRC cells recruit CCR1+ cells through the CCL15–CCR1 signaling to promote liver metastasis in a mouse model

To investigate whether CCL15 expression by human CRC cells promotes their metastasis to the liver in transplanted mice, we injected luciferase-expressing CRC cells into spleens of nude mice, which enabled monitoring of the disseminated and metastasized tumor cells in the liver by bioluminescence. This method is also useful to quantify the metastasized tumor cells by photon counts (Supplementary Fig. S3A). We used DLD-1, HCT116 and HT29 cells (Luc-DLD-1, Luc-HCT116 and Luc-HT29,

respectively) engineered with stable *SMAD4*-knockdown (sh*SMAD4*) or with stable Smad4-overexpression (see Methods). Like the siRNA data in Fig. 1C above, both sh*SMAD4* constructs (sh*SMAD4*#1 and sh*SMAD4*#2) decreased Smad4 expression, and increased *CCL15* level in Luc-DLD-1 and Luc-HCT116 cells (Fig. 3A left; Fig. 3B left and center), whereas Smad4 overexpression reduced CCL15 expression in Luc-HT29 cells (Fig. 3A right and 3B right). The proliferation rates of these cells were not affected in any of the cell lines (Supplementary Fig. S3B). Next, we assessed these transfectant clones in nude mice for liver metastasis. When injected with Luc-DLD-1/HCT116 sh*SMAD4* cells, their bioluminescence intensity markedly increased compared with those with the control (Fig. 3C left and center; Fig. S3D left and center). In contrast, mice with Luc-HT29 cMyc-Smad4 cells showed a significant reduction in the liver bioluminescence compared with those with the control (Fig. 3C right and Fig. 3D right). Notably, mice with Smad4-depleted cells (Luc-DLD-1/HCT116 sh*SMAD4* and Luc-HT29 cMyc-tag) showed substantially shorter survival than those with Smad4-expressing cells (Luc-DLD-1/HCT116 scramble or Luc-HT29 cMyc-Smad4) (Supplementary Fig. S3C).

We next histologically examined the mouse livers containing metastatic CRC cells. Liver tumors of Smad4-deficient cells (Luc-DLD-1/HCT116 sh*SMAD4* and Luc-HT29 cMyc-tag) expressed CCL15, whereas those with Smad4-expressing cells (Luc-DLD-1/HCT116 scramble and Luc-HT29 cMyc-Smad4) did not (Fig. 3E). Consistently, Smad4-deficient tumor cells contained CCR1+ cells and MMP9+ cells around the liver metastases, whereas Smad4-expressing tumor cells did not (Fig. 3E). These results indicate that loss of Smad4 in CRC cells promotes CCL15 expression to recruit CCR1+ cells in the liver, which causes metastatic colonization of tumor cells disseminated to the mouse liver.

Expression level of CCL15 is inversely correlated with that of Smad4 in human liver metastases of CRC

We previously reported that the expression level of CCL9 was increased in intestinal

adenocarcinomas of *cis-Apc/Smad4* mice.[15] As shown in Fig.1–3, we have found that Smad4 negatively regulates expression of CCL15 in human CRC cell lines, and that loss of Smad4 promotes liver metastasis of CRC through CCL15–CCR1 signaling in a mouse model. Therefore, we next examined clinical specimens of human CRC liver metastases by immunohistochemistry (IHC), and investigated whether the CCL15 level was elevated (Fig. 4A). Of 141 liver metastases obtained from 67 operations of 55 CRC patients (Table 1), we found that 63% (89 of 141) was negative for Smad4, whereas 37% (52 of 141) was positive, which was consistent with the frequency previously reported in advanced stage CRC.[9-11] Expression of CCL15 was found in 79% (70 of 89) of the Smad4-negative metastases, whereas it was in 37% (19 of 52) of the Smad4-positive metastases, with a significant inverse correlation between Smad4 and CCL15 expression (odds ratio, 0.16; $P < 0.01$, Table 2). To evaluate the clinical significance of the CCL15 expression in CRC, we analyzed the disease-free survival (DFS) for the 44 patients who underwent curative liver resection without metastasis to any other organs such as lungs. Statistical analysis by the log-rank test showed that the patients with CCL15-expressing liver metastases had a significantly shorter DFS than those with CCL15-negative metastases ($P = 0.04$, Fig.4B). In these 44 patients, the number of liver metastases was higher in the CCL15-positive lesions than in the CCL15-negative ones, although the difference was not statistically significant ($P = 0.09$, Supplementary Table S5). On the other hand, there was no substantial difference in other factors such as preoperative CEA levels and primary tumor size (Supplementary Table S5). 31 patients experienced recurrence after curative liver resection, most of which occurred in the liver (84%; 26 in 31 patients).

Taken together, we conclude that loss of Smad4 in CRC is associated with expression of CCL15 and poorer prognosis in human CRC as in mouse models.

CCL15 recruits CCR1+ myeloid cells to metastatic microenvironment of human liver

Using mouse models, we previously reported that CCR1+ myeloid cells expressing MMP9 accumulated around the primary and metastatic CRC lesions that secreted CCL9, which promoted cancer invasion and metastasis to the liver.[15,16] To confirm the clinical relevance of these findings, we performed further IHC. Notably, significant numbers of CCR1+ cells accumulated around the CCL15-expressing CRC cells in the liver, but few around the CCL15-negative cells (Fig. 4A). We quantified the density of CCR1+ cells at the periphery of liver metastases (Supplementary Fig. S4A), and found ~3 times more CCR1+ cells around the CCL15-positive metastases than around CCL15-negative ones (34.3 ± 18.6 vs. 10.3 ± 8.8, respectively; $P < 0.01$, Table 2; Fig. 4C). We also found that the CCR1+ cell density was inversely correlated with the metastatic tumor size ($P < 0.01$, Fig. 4D). Namely, the smaller the liver metastases, the higher numbers of CCR1+ cells were found around the tumors, which is similar to the observation in a mouse model.[16] To characterize the CCR1+ cells, we determined their expression of CD11b, myeloperoxidase (MPO), MMP9 and CD34 by immunofluorescence staining (Fig. 5). Because Gr-1 is not expressed in humans, we employed CD11b and MPO as human myeloid cell markers.[29] We found that the CCR1+ cells were positive for both CD11b and MPO (Fig. 5A, 5B and 5C), suggesting that they are of the myeloid origin. Moreover, these CCR1+ myeloid cells also expressed MMP9 (Fig. 5A and 5D), which is consistent with the data obtained from the mouse models. Although CCR1+ myeloid cells were positive for CD34 in mouse models, co-staining with CCR1 and CD34 on human samples revealed that CCR1+ cells were negative for CD34 (Fig. 5E). This discrepancy among these results could be due to the difference of developmental stages of myeloid progenitor cells between the human and mouse.[30] To understand the chronology of CRC metastasis, we investigated 49 primary CRC, and found that CCL15 expression within the primary CRC was correlated with lack of Smad4 expression, CCR1+ cell accumulation and liver metastasis (Supplementary Fig. S4B and Supplementary Table S6). These data strongly suggested that recruitment of CCR1+ cells by CCL15 is a feature of a human CRC subset, as well as in mice.

Taken together, these results indicate that loss of Smad4 promotes expression of CCL15 in metastatic CRC cells to recruit CCR1+ myeloid cells, which can facilitate early metastatic expansion in humans as in the mouse model (Fig. 5F).

Discussion

SMAD4 is one of the genes in human chromosome 18q21 where frequent LOH is observed in CRC progression as a well-known poor prognostic marker.[9,11,31] Although loss of Smad4 is implicated in the progression of CRC,[6–8] the molecular mechanisms remain to be elucidated. Here we have shown that loss of Smad4 increases expression of CCL15 in human CRC and causes tumor-stromal interaction through the CCL15–CCR1 signaling (Fig. 5F). There is an inverse correlation between the levels of Smad4 and CCL15 in both human CRC cell lines and clinical specimens. We have further confirmed that Smad4 binds directly to the promoter region of *CCL15* gene to negatively regulate its expression through the TGF-β and BMP signaling. In the TGF-β family signaling, several transcriptional co-repressors recruit histone deacetylases (HDACs) to the Smad complexes, which leads to transcriptional repression of the target genes.[32] We have found that Smad4 binds to HDAC4/5, and that a HDAC inhibitor, Trichostatin A, promotes *CCL15* expression in CRC cell lines (Supplementary Fig. S2F and S2G). Analysis of human clinical specimens showed that CCL15-expressing CRC, most of which lacked Smad4 expression, contained higher density of CCR1+ myeloid cells in the primary tumors and liver metastases, as observed in a mouse model.[15,16] Interestingly, the smaller the liver metastases, the more CCR1+ cells accumulated around tumors, suggesting that CCR1+ myeloid cells help CRC cells to colonize liver metastasis at early stages. Recently, it has been reported that BMPs, members of the TGF-β superfamily, directly regulate CCL9 expression through Smad1/5/8 in mouse mammary tumors.[33] We have also found that expression of CCL15 was decreased with BMP4 stimulation in human CRC cell lines (Supplementary Fig. S1E and S1F).

In addition to the cell-autonomous changes in cancer cells, there is mounting evidence that the stromal cells, e.g. BMDCs, play key roles in malignant progression.[34,35] It has been reported that hematopoietic progenitors that express CD11b and vascular endothelial growth factor receptor 1 (VEGFR1) accumulate at the premetastatic niche in lungs.[12] It has been also reported that VEGFR1 and CXCR4 independently exert a

promigratory effect in BMDC through the VEGF/VEGFR1 and CXCL12/CXCR4 pathways, respectively, in mouse models.[36] We previously reported that CCR1+ myeloid cells accumulated around the primary and metastatic lesions of CRC through the mCCL9/hCCL15–CCR1 signaling in mouse models.[15,16] Likewise, it was reported in a breast cancer mouse model that loss of TGF-β signaling increased CXCL1 and CXCL5 secretion, which increased migration of CD11b+Gr-1+ myeloid-derived suppressor cells (MDSCs) to the tumor tissues,[13] and played indirect roles in increasing the number of Th17 cells that have a protumorigenic effect.[37] Taken together, different subsets of BMDCs may be involved in the invasion and metastasis of a variety of tumors through different chemokines. However, most these findings were obtained from mouse models, and it has remained to be investigated whether similar mechanisms are actually involved in humans. In this study, we have demonstrated that significantly larger numbers of CCR1+ myeloid cells are detected in CRCs expressing CCL15 than in those not expressing CCL15. We have also found that the patients with CCL15-expressing liver metastases have shorter DFS than those with CCL15-negative tumors with a statistical significance. Accordingly, blocking the CCL15–CCR1 axis can be an efficacious strategy for CRC patients to prevent liver metastasis. Larger cohort studies using CCR1 inhibitors may confirm the role of CCL15–CCR1 axis in liver metastasis of CRC.

There are some CCR1 inhibitors already used in phase I/II clinical trials to the patient with arthritis,[38] multiple sclerosis,[39] or chronic obstructive pulmonary disease (COPD).[40] These inhibitors have already been tested for their safety although their efficacies in those targeted diseases remain to be seen. So far, there has been no report of CCR1 inhibitors employed for an anti-cancer treatment. Using a mouse model for CRC liver metastasis, we previously reported that a CCR1 antagonist blocked the CCR1+ cell accumulation and metastatic colonization with significantly prolonged survival of the tumor-bearing mice.[16] Therefore, selectively targeting CCR1+ myeloid cells, perhaps in combination with conventional chemotherapeutics, can be a novel strategy against CRC patients with liver metastases.

Legends to Figures

Figure 1. Expression of CCL15 and Smad4 in human CRC and adenoma cell lines. (A) Western blot analysis showing protein expression of Smad1, Smad2/3, Smad5, Smad4, CCL15 and β-actin (ACTB). (B) Quantitative RT-PCR analysis showing mRNA levels for *CCL15*. (C and D) Western blot (C) and quantitative RT-PCR (D) analyses showing Smad4 levels in Smad4-expressing CRC cell lines. Nt and Ns indicate non-treatment and non-silencing scramble siRNA as controls. (E and F) Western blot (E) and quantitative RT-PCR (F) analyses showing expressions of CCL15 and Smad4 in Smad4-deficient cell lines. Cells were treated with or without 10 ng/ml TGF-β1 for 24 h (E) or 12 h (F). The signal band strengths of western blot for CCL15 were quantified using Image Lab software (Bio-Rad). Results are presented as the means ± SD of triplicate measurements (*$P < .01$. **$P < .05$)

Figure 2. Smad4 directly binds to the *CCL15* promoter and negatively regulates its expression through TGF-β signaling. (A) Schematic representation of the *CCL15* promoter region. The sequence of this region was obtained by sequencing genomic DNA of HCT116, and matches with that of database obtained from UCSC genome browser.[41] Transcription start site (black arrow) was confirmed by 5' rapid amplification of cDNA end (5'RACE). (B) Luciferase reporter assay for the *CCL15*-Luc activity with or without Smad4 knockdown in Smad4-expressing cell lines. Transcription activities of the *CCL15* promoter are represented as the ratio *CCL15*-Luc/pGL4. (C) Luciferase reporter assay for the *CCL15*-Luc activity with or without stable Smad4 overexpression in Smad4-deficient cell lines in the presence or absence of TGF-β1. (D) Luciferase reporter assay for the *CCL15*-Luc deletion constructs. There are typical TATA box motif (+41) and downstream promoter element (DPE; GACTC starting +97) in the *CCL15* promoter, and deletion of the 4th SBE (Δ4th SBE) extended into this DPE, which may be the reason why the Δ4th SBE mutant showed reduced luciferase activity.[42] (w.t., wild-type). (E and F) ChIP-PCR analysis for enrichment of each SBE or TIE in the *CCL15* promoter region

(HCT116 in (E) and HT29 cMyc-Smad4 with 10 ng/ml TGF-β1 stimulation in (F)). DNA samples were analyzed by quantitative PCR and adjusted to 10% input. Western blots show Smad4 with 10% inputs and immunprecipitated samples (right). Results are presented as the means ± SD of triplicate measurements (*$P < .01$, **$P < .05$).

Figure 3. Mouse xenograft models of CRC liver metastasis. (A and B) Western blot (A) and quantitative RT-PCR (B) analyses of luciferase-expressing CRC cell lines where *SMAD4* was stably knocked-down (in Smad4-expressing cells; Luc-DLD-1 and Luc-HCT116) or overexpressed (in Smad4-deficient cells; Luc-HT29). (C) *In vivo* bioluminescence images of mice injected with luciferase-expressing CRC cells. (D) Quantification of the liver metastatic lesions (photon counts) in mice injected with the cells described in Supplementary Fig S3A. n = 3 for each group. (E) IHC analysis of mouse livers with xenograft CRC cell lines. Scale bar, 100 μm. Results are presented as the means ± SD of triplicate measurements (*$P < .01$).

Figure 4. Expression of CCL15 and recruitment of CCR1+ cells in the liver metastatic lesions of CRC patients. (A) Haematoxylin and eosin staining (H & E) and IHC staining for Smad4, CCL15 and CCR1 of liver specimens from metastatic CRC patients. Upper and lower panels show serial sections of representative Smad4-negative and Smad4-positive liver metastases, respectively. Scale bar, 100 μm. (B) Effects of CCL15 expression on DFS in patients who underwent curative liver resection without any other distant metastases. (Kaplan-Meier estimates) (C) Quantification of the CCR1+ cell density in CRC liver metastases with and without CCL15 expression (n=89 and 52, respectively). (*$P < .01$; exact Mann-Whitney *U* test; horizontal bands show the means). (D) Scatter plot of tumor size and CCR1+ cell-accumulation. The relationship was evaluated by the Pearson's correlation coefficient (r; correlation coefficient).

Figure 5. Characterization of CCR1+ cells in the microenvironment of CRC liver

metastases. (A) IHC staining for CCR1, CD11b, MPO and MMP9. Scale bar, 100 μm. (B–E) Immunofluorescence staining for CCR1 and CD11b (B), MPO (C), MMP9 (D), and CD34 (E). Scale bar, 20 μm. (F) Schematic representation of the CCL15-CCR1 axis in liver metastasis of CRC. In CRC cells, Smad4 inhibits the expression of CCL15 downstream of TGF-β and BMP signaling (left). Once Smad4 is inactivated, this inhibition is relieved and CCL15 is expressed. Chemokine CCL15 recruits CCR1+ myeloid cells around metastatic CRC cells. The bone marrow-derived CCR1+ cells produce MMPs and facilitate tumor expansion (center and right).

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Author names in bold designate shared co-first authorship.

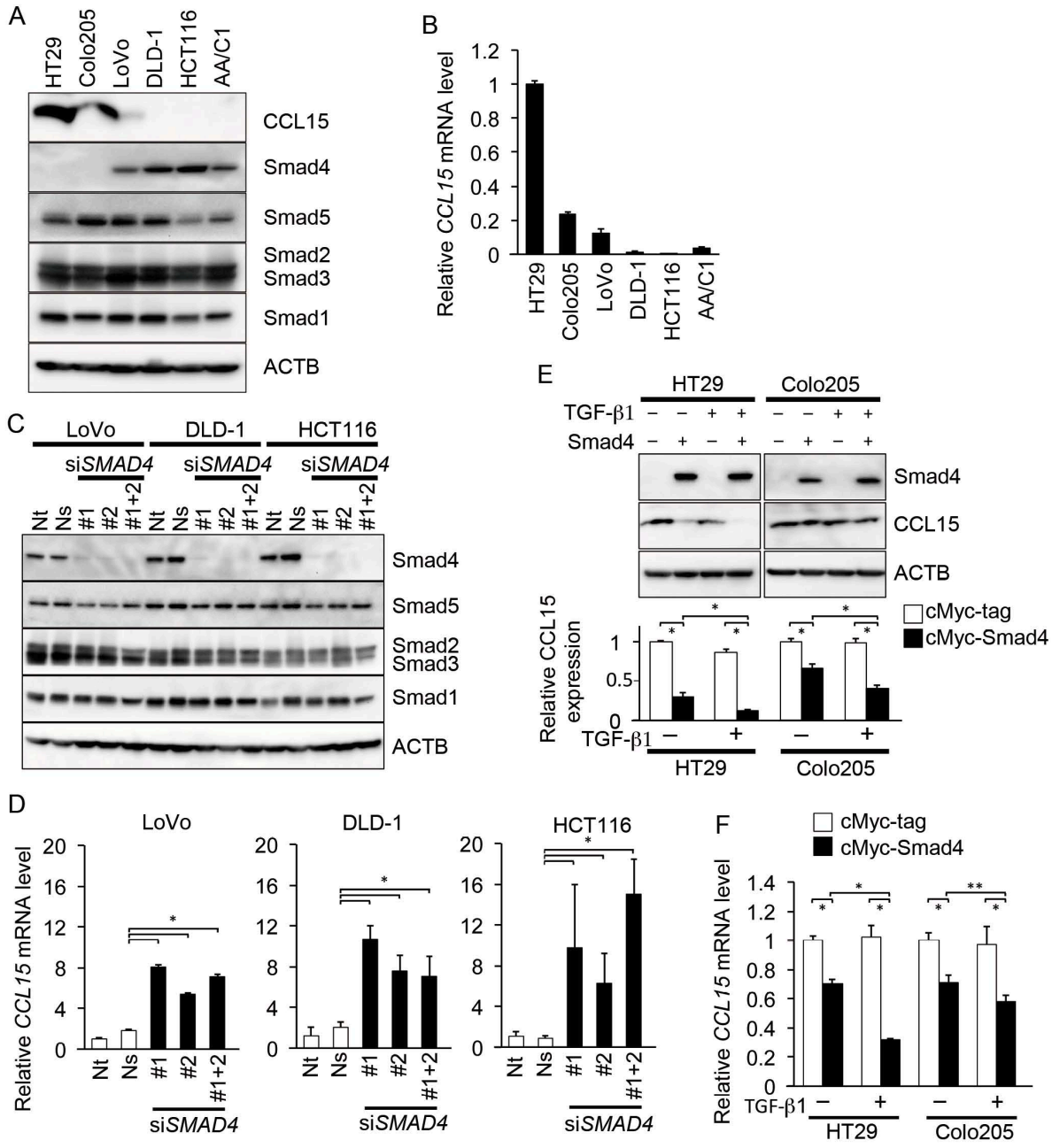
Table 1. Patient and Tumor Characteristics (141 Metastases Obtained from 67 Operations of 55 Patients)

Characteristics	Patients	Resections
Age, years		
Mean ± SD	64.8 ± 10.9	
Range	36–87	
Sex		
Male	33	
Female	22	
Location of primary tumor		
colon	37	
rectum	18	
Resection times		
One	44	
Two	10	
Three	1	
Timing		
Synchronous		21
Metachronous		46
CEA		
< 5.0		20
≥ 5.0		47
No. of metastases		
1		29
2		20
3		6
4		7
5		4
6		1
Prior chemotherapy		
No.		39
Yes.		28

Table 2. Univariate Analysis of Liver Metastases of CRC with Factors Associated with CCL15 Expression

	CCL15 expression		
Factors	+ (n = 89)	− (n = 52)	*P*-value
Maximum tumor size (ϕ in mm)	21.6 ± 20.3	23.0 ± 19.1	.69
Smad4 expression			< .01
Positive	19	33	
Negative	70	19	
$CCR1^+$ cell count	34.3 ± 18.6	10.3 ± 8.8	< .01

Figure 1



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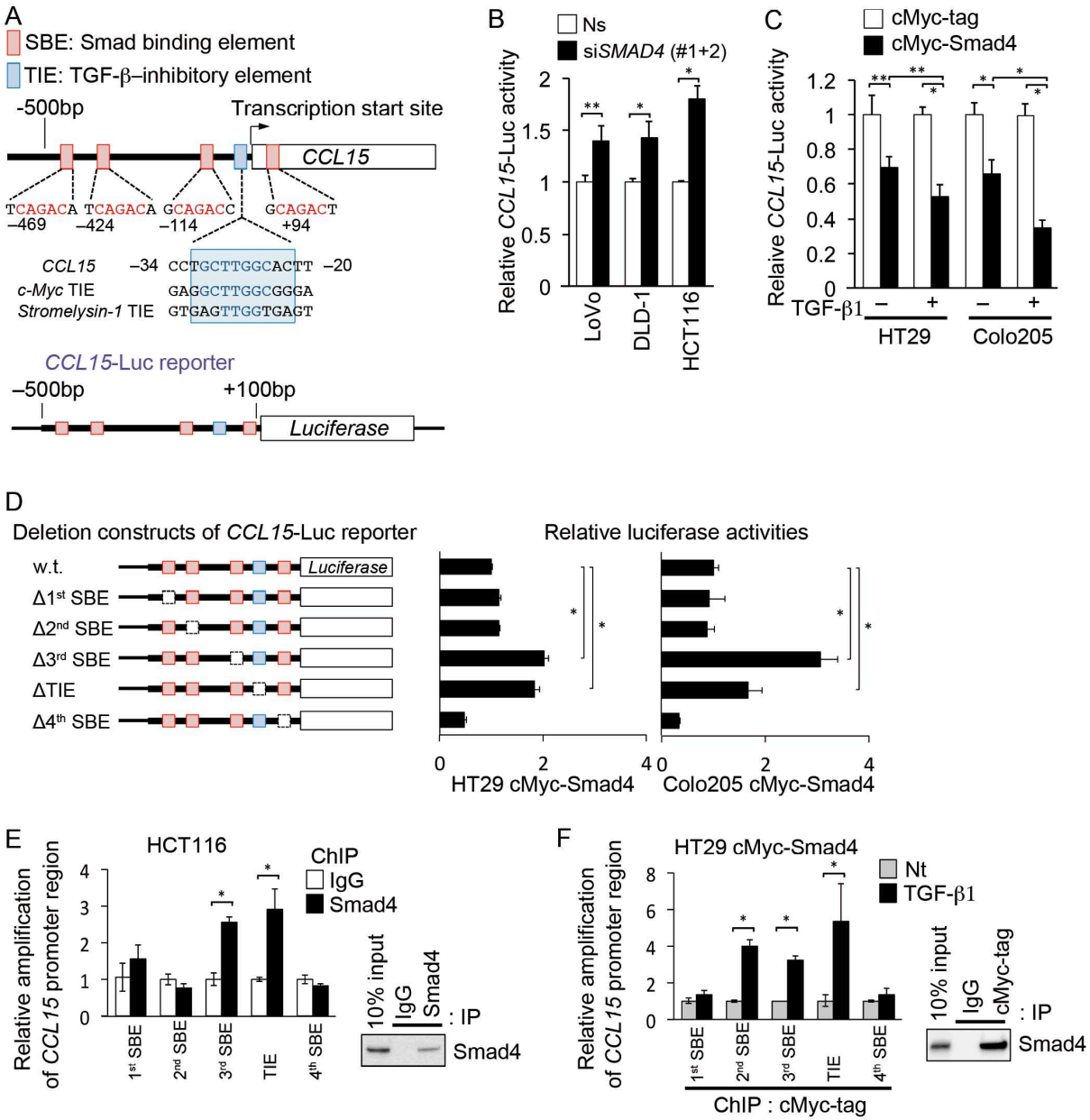

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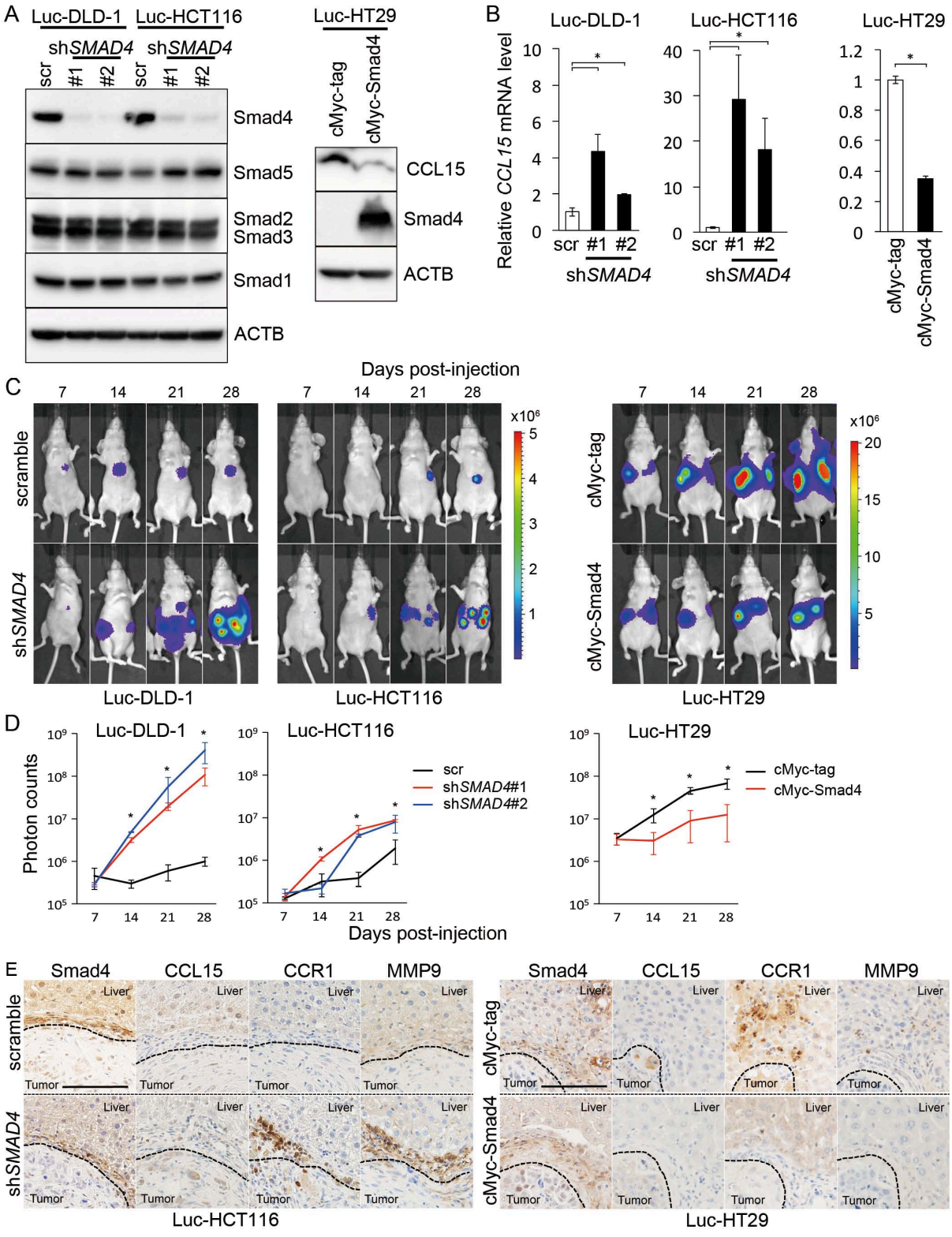


Figure 4

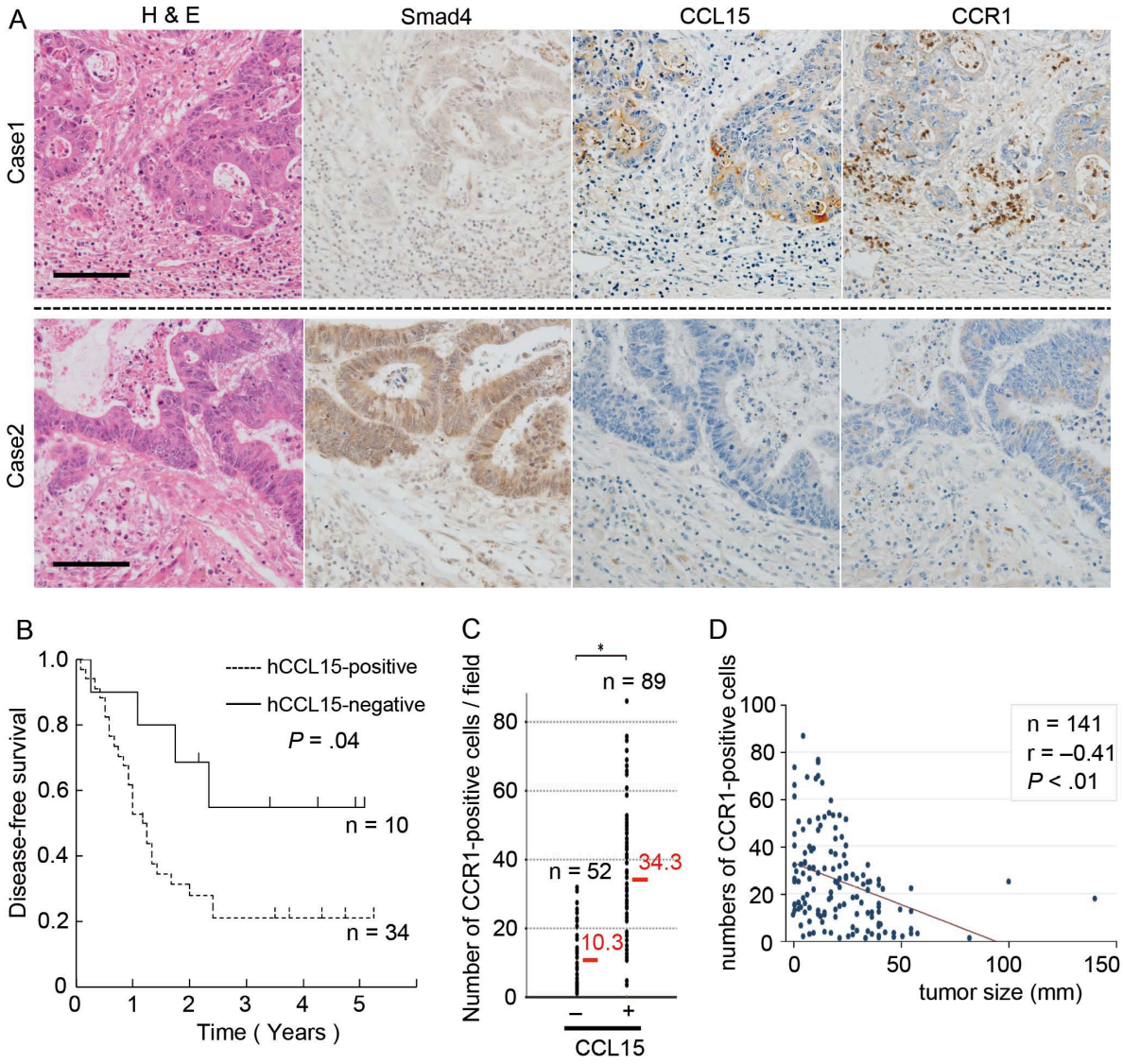
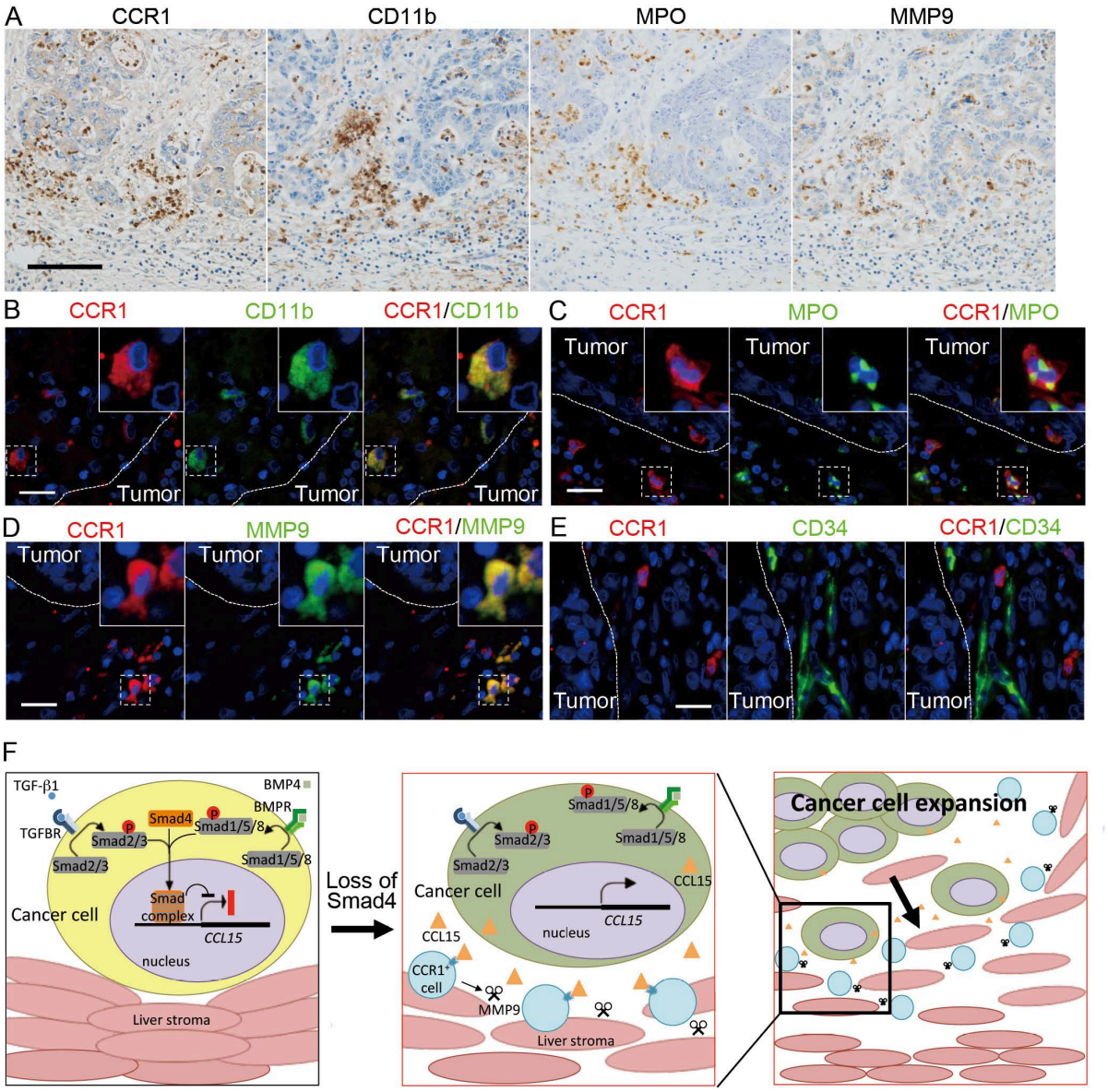
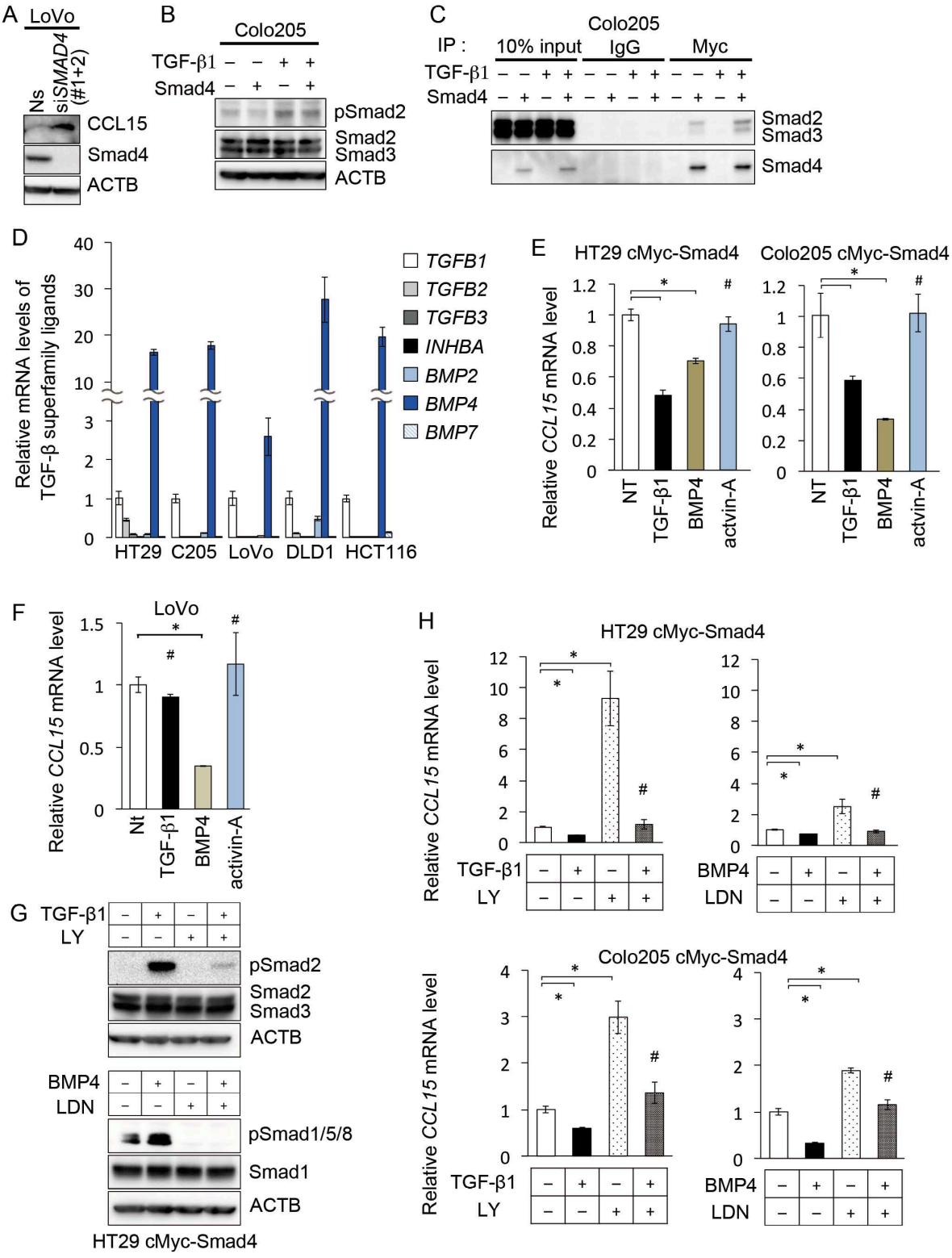


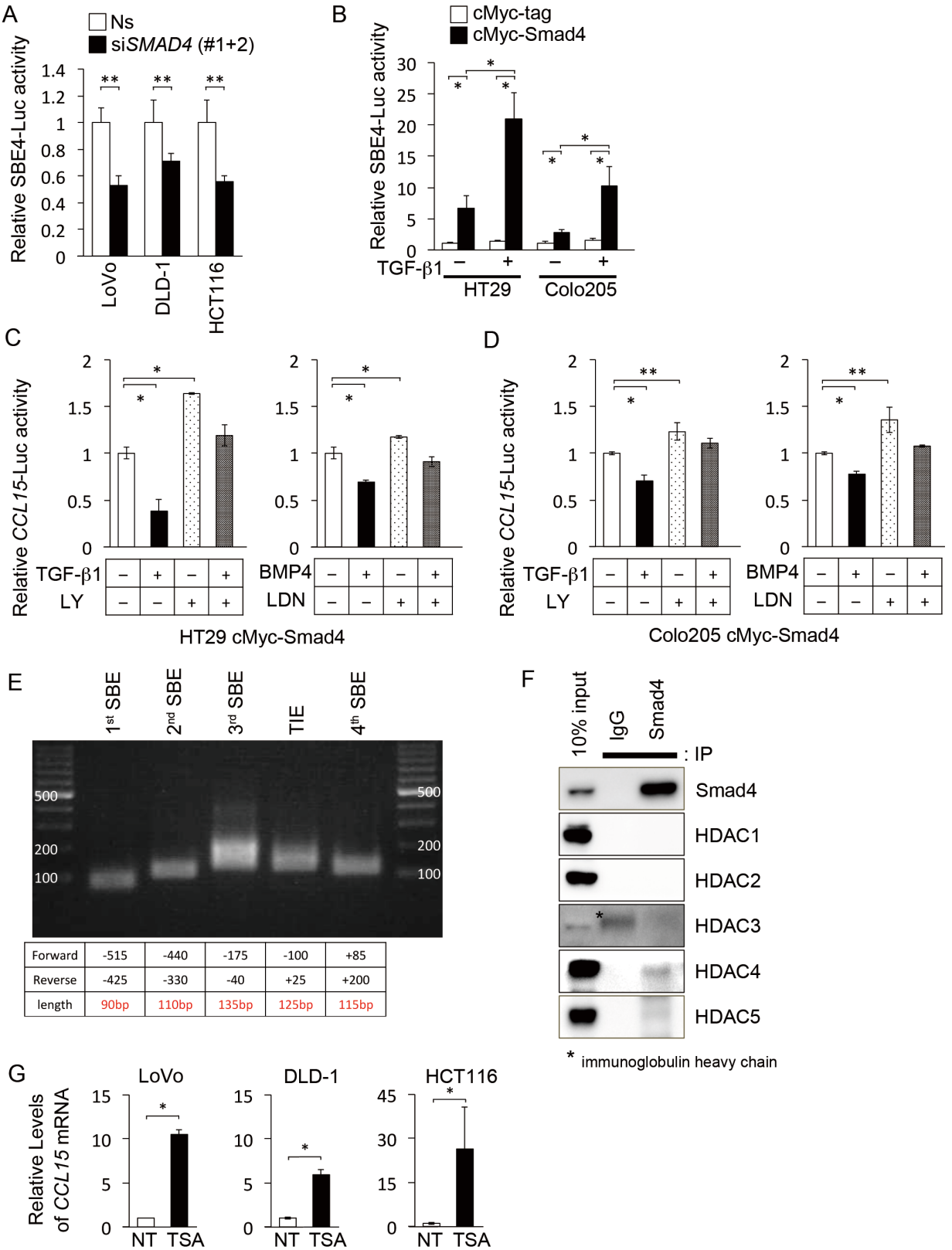
Figure 5



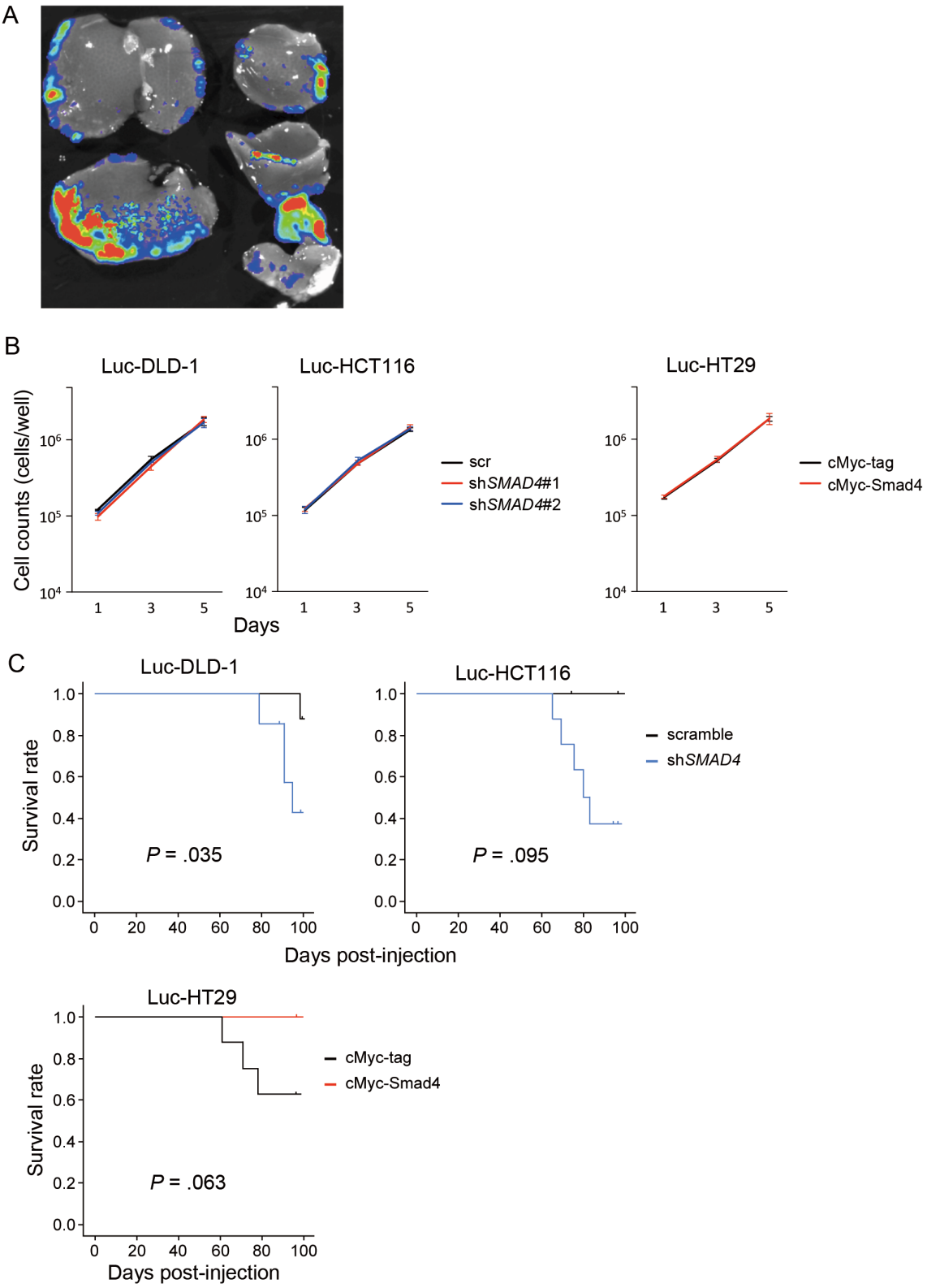
Supplementary Fig S1



Supplementary Fig S2

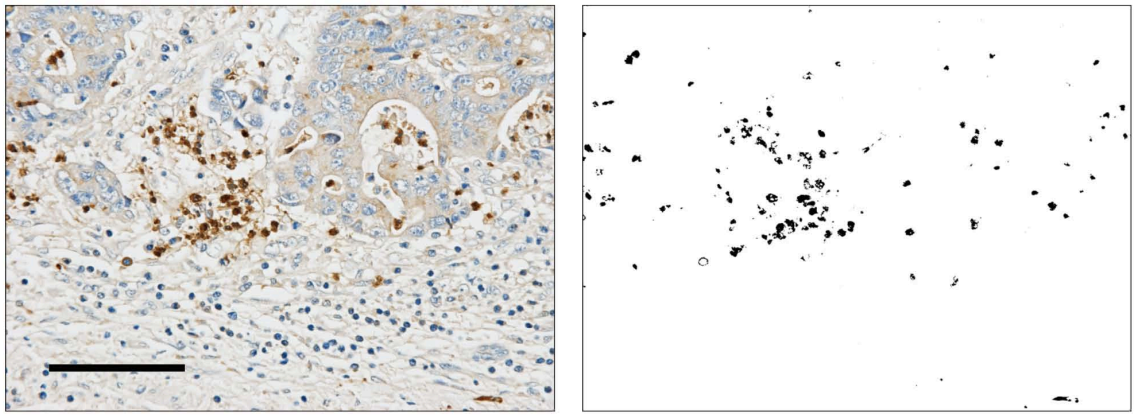


Supplementary Fig S3



Supplementary Fig S4

A



B